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Sulfur mustard disrupts human $\alpha_3\beta_1$-integrin receptors in concert with $\alpha_6\beta_4$-integrin receptors and collapse of the keratin K5/K14 cytoskeleton

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ABSTRACT

Sulfur mustard (SM; bis(2-chloroethyl) sulfide) is a chemical warfare agent that produces persistent, incapacitating blisters of the skin. The lesions inducing vesication remain elusive, and there is no completely effective treatment. Using multiphoton microscopy and immunofluorescent staining, we found that exposing human epidermal keratinocytes (HEK) and intact epidermis to SM (400 μM for 5 min) caused progressive collapse of the keratin (K5/K14) cytoskeleton and depletion of $\alpha_6\beta_4$ integrins.[1] We now report that SM causes concomitant disruption and collapse of the basal cell's $\alpha_3\beta_1$-integrin receptors. At 1 h postexposure, images of Alexa488-conjugated HEK/$\alpha_3\beta_1$ integrins showed almost complete withdrawal and disappearance of retraction fibers and a progressive loss of polarized mobility. With stereo imaging, in vitro expression of this SM effect was characterized by collapse and abutment of adjacent cell membranes. At 2 h postexposure, there was an average 13% dorso-ventral collapse of HEK membranes that paralleled progressive collapse of the K5/K14 cytoskeleton. $\alpha_3\beta_1$ integrin, like $\alpha_6\beta_4$ integrin, is a regulator of cytoskeletal assembly, a receptor for laminin 5 and a mediator of HEK attachment to the basement membrane. Our images indicate that SM disrupts these receptors. We suggest that the progressive disruption destabilizes and potentiates blistering of the epidermal-dermal junction.

Keywords: $\alpha_3\beta_1$ integrins, sulfur mustard, blisters, human epidermal keratinocytes

1. INTRODUCTION

Sulfur mustard (SM) is a percutaneous, fast acting alkylating agent capable of producing pre-vesicating lesions in human skin within 2 minutes of topical exposure.[2] The cytopathology produced by SM is typically progressive and elicits incapacitating blisters after a dose-dependent clinical latent phase of 8-24 hours. This agent has been a weapon of war for over 85 years, and the global threat of hostile exposure to SM is greater today than at any time since World War I. Efforts to develop treatment strategies have been confounded by not knowing precisely how human skin is induced by SM to produce blisters. We know that genetic disorders effecting altered assembly of keratins K5/K14 can cause epidermolysis bullosa simplex and blisters that occur in basal cells above their hemidesmosomes.[3,4] However, the blisters produced by SM are typically located at the epidermal-dermal junction.[5,6] Their phenotype resembles blisters produced by junctional epidermolysis bullosa and by mutations that disrupt assembly of $\alpha_3\beta_1$ integrins[7] or $\alpha_6\beta_4$ integrins and laminin 5.[8] With multiphoton microscopy [9,10] and immunofluorescent imaging techniques, we are beginning to unravel the mystery of SM toxicity. In the following study, images show how $\alpha_3\beta_1$ and $\alpha_6\beta_4$ integrin receptors are disrupted by SM in concert with collapse of the keratin K5/K14 cytoskeleton and how these lesions might induce blistering.

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2. MATERIALS AND METHODS

Sec. 2.1 Human epidermal keratinocytes (HEK)

Replicate cultures of human epidermal keratinocytes (Clonetics® NHEK; Cambrex Bio Science, Walkersville, MD) were grown on borosilicate glass coverslips in keratinocyte growth medium (KGM; Cambrex) at 37 °C in a humidified atmosphere of 5% CO_2 and air. HEK were maintained in exponential growth to densities of ~50,000 cells/cm^2 and were approximately 70% confluent at experimentation.

Sec. 2.2 Human epidermal tissues

Mammary skin explants from the Cooperative Human Tissue Network (CHTN; Ohio State University, Columbus, Ohio) were processed as previously described[11] by cutting away the excess adipose and connective tissue from the ventral surface and incubating cm^2 sections in dispase (10 U/ml in PBS/KGM) overnight at 6 °C. Epidermal tissues were detached immediately prior to use.

Sec. 2.3 Sulfur mustard exposures

Replicate HEK cultures and epidermal tissue sections were exposed for 5 min to fresh KGM (at 37 °C) for control studies and to KGM containing 400 μM SM for experimental studies. HEK and epidermal tissues were washed 3x and replenished with fresh KGM before returning them to the CO_2 incubator.

Sec. 2.4 Fixing and staining of HEK and epidermal tissues

At 1-h intervals after SM exposure, HEK were fixed with 4% paraformaldehyde (10 min at room temperature) for the study of $\alpha_3\beta_1$-integrin receptors and were postfixed for 3 min with 100% acetone at –20 °C for the study of keratin 14. Epidermal tissues were fixed overnight in a modified Karnovsky's fixative containing 1.6% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4. For $\alpha_3\beta_1$ studies, HEK and epidermal tissue preparations were washed 3x in PBS and incubated 1.5 h at room temperature in a 1: 50 PBS dilution of mouse anti-human $\alpha_3\beta_1$ integrin, Clone P1B5 (DAKO Corp., Carpintera, CA). Preparations were then washed 3x (3 min each) with PBS and incubated 1.5 h in a 1:50 dilution of Alexa488-conjugated goat anti-mouse IgG (H & L) (Molecular Probes Inc., Eugene, OR). Keratin 14 and $\alpha_6\beta_4$ integrin studies were conducted as previously described.[1] All preparations were subjected to a final wash (3x in PBS) prior to imaging as wet mounts.

Sec. 2.5 Multiphoton imaging

Imaging was performed with an MRC-1024 multiphoton laser scanning system (Bio-Rad, Hemel Hempstead, UK) using an Axiovert-135 inverted microscope and a 63x/1.2 NA water objective (Carl Zeiss Inc., Thornwood, NY). Excitation was conducted at 780 nm using a Verdi 5-watt diode pump and a Mira 900 Ti:sapphire pulsed laser with X-wave optics (Coherent Laser Group, Santa Clara, CA). Emissions were directed to external detectors through filter sets (Chroma Technology Corp., Brattleboro, VT) selected to enhance our imaging signals.

Sec. 2.6 Image analyses

Analyses of optical sections, 3-D reconstructions and stereo images were performed using Bio-Rad's LaserSharp software.

3. RESULTS

Postexposure imaging of fluorescently tagged human epidermal keratinocytes showed that a 5-min exposure to SM caused a progressive collapse of HEK and their $\alpha_3\beta_1$ surface receptors. The early onset, sequellae and characteristic features of the HEK/$\alpha_3\beta_1$ collapse are presented (figure 1) in 3-D stereo images (A-C) and in complementary optical sections (D-F) taken at 1.4 μm from the HEK attachment surface. Note: *Use 3-D glasses to enhance viewing of the stereo images. Place the green lens over your right eye for normal viewing and red lens over your right eye for inverted viewing.* In control populations, HEK/$\alpha_3\beta_1$ integrins formed a

thin, veil-like cover that overlaid the raised central nucleus or dome of each cell and flowed out and down the surface of each cell to its narrow rim of basolateral attachments (figure 1A). Cells often contained an open, well-defined ring (aperture) within the veil of $\alpha_3\beta_1$ receptors. HEK/$\alpha_3\beta_1$ integrins were also expressed in the filopodial retraction fibers of mobile cells (figure 1D). At 1 h postexposure, emissions from $\alpha_3\beta_1$ integrins were more intense at the cell margins. Stereo images revealed that the bright emissions resulted from lateral compression of adjacent cell membranes and accretion of their $\alpha_3\beta_1$ surface receptors (figure 1B). Optical sections taken near the HEK attachment surface showed an obvious loss of retraction fibers and functional asymmetry (figure 1E). However, HEK did maintain an otherwise characteristic $\alpha_3\beta_1$ surface appearance. At 2 h postexposure, HEK had a non-motile, flagstone appearance and collapse of their domed centers was evident (figure 1C). Optical sections taken near the HEK attachment surface were absent of retraction fibers and had lost all signs of functional asymmetry (figure 1F). Despite this pathology, the dorsal appearance of HEK/$\alpha_3\beta_1$ surface receptors looked relatively intact.

Analysis of image profiles from control and exposed populations showed that an acute 5-min exposure to SM induced a progressive, postexposure collapse of HEK and, accordingly, of their $\alpha_3\beta_1$-integrin receptors. By quantitative comparison of their image sections, we determined that the average height of HEK ($\pm$ S.E.M.) decreased at 1 h postexposure from 10.75 $\pm$ 0.10 µm to 9.94 $\pm$ 0.19 µm, i.e., by 7.5% compared with replicate controls (n = 44). By 2 h postexposure, the collapse of HEK had worsened, and the average image height of HEK/$\alpha_3\beta_1$ integrins decreased from 9.03 $\pm$ 0.21 µm to 7.84 $\pm$ 0.18 µm, i.e., by 13.2% when compared with replicate controls (n = 34). The graphics of postexposure HEK/$\alpha_3\beta_1$ collapse (figures 2A & 2B) indicate that the effect is coincident with the progressive, postexposure collapse of the cell's keratin K5/K14 cytoskeleton, which has been recorded in previous SM studies. Based on images from our keratin studies (figure 3), the collapse of keratin 14 at 1 h postexposure appeared to be slightly more advanced than that of the HEK/$\alpha_3\beta_1$ surface integrins. At 2 h postexposure, collapse of the K5/K14 cytoskeleton and the HEK/$\alpha_3\beta_1$ surface integrins were more nearly alike (quantitative comparisons not shown). Previous studies[1] showed that collapse of the K5/K14 cytoskeleton continued to worsen through 6 h of postexposure study.

In optical sections from the ventral surface of intact human epidermis, the images of tissues stained for $\alpha_3\beta_1$ integrins revealed a necklace of bright, evenly spaced, punctate units that outlined the perimeter of the constituent basal cells (figure 4A). Stereo imaging of the same image fields showed that the punctate units were, in fact, cross sections of $\alpha_3\beta_1$-integrin tracts that ran parallel to the long axis of each cell (figure 4B). The arrangement of those tracts and their dorso-ventral presentation of $\alpha_3\beta_1$ receptors was perpendicular to the $\alpha_6\beta_4$ integrin receptors on the ventral surface of the polarized basal cells (figures 4C and 4D). The distinctly different and inherent distribution of these two adhesion-receptor types obviously provides different aspects for outside-in and inside-out communication between basal cells and their extracellular environment and, therefore, different aspects for cell attachment and signal transduction. Multiphoton imaging has shown that SM disrupts the organization and presentation of both these receptor types in culture and in intact epidermal tissues (figures 1 and 4E). The disruptive effect on both receptors was progressive and while the phenotypic expression of their response differed, both effects were coincident with progressive postexposure collapse of the K5/K14 cytoskeleton.

4. DISCUSSION

Multiphoton imaging is helping to define how SM disrupts the structural and functional integrity of $\alpha_3\beta_1$ integrins and the dermal-epidermal attachments of basal keratinocytes. Within the basal cell's adhesion complex, $\alpha_3\beta_1$ and $\alpha_6\beta_4$ integrins are both receptors for laminin 5.[12,13] We know that $\alpha_6\beta_4$ and laminin 5 are early targets of SM[1] and that disruption of these molecules can cause blistering at the dermal-epidermal junction. Although $\alpha_3\beta_1$ and $\alpha_6\beta_4$ integrins share a common function as laminin-5 receptors, they are clearly recruited to distinct cell attachment sites (figure 4) and are uniquely organized to perform separate but complementary functions. For example, the $\alpha_3\beta_1$ integrins of the basal epidermis are organized into polarized, peripheral tracts that run parallel to the long axis of the constituent cells. Their focal contacts link

the extracellular matrix (ECM) to the actin cytoskeleton.[14-17] The $\alpha_6\beta_4$ integrins cover the basal cell's ventral surface with a network of receptors that link the ECM (basement membrane) to the K5/K14 cytoskeleton. These integrins ($\alpha_3\beta_1$ and $\alpha_6\beta_4$) serve to stabilize the epidermal-dermal junction, and facilitate organization of their laminin-5 ligand for cell migration and wound repair;[18] they also play an important role in signal transduction.[19] Our images show that SM disrupts the structural integrity of these interactive molecules. Our results indicate that dorso-ventral collapse and lateral compression of the $\alpha_3\beta_1$ receptors was coincident with progressive collapse of the keratin K5/K14 cytoskeleton and loss of functional asymmetry (polarized mobility). We suggest that the early disruption of $\alpha_3\beta_1$ integrins and related adhesion complex molecules are linked events that interfere with signal transduction and exacerbate the progressive loss of structural and functional integrity. The combined effect would destabilize the epidermal-dermal junction, potentiate vesication and deprive basal cells of anchorage dependent functions, e.g., growth, motility, repair and ultimately, viability. Our image profiles also indicate that the window of opportunity for effective treatment and resolution of SM toxicity is limited by the early onset and progressive nature of the lesions produced.

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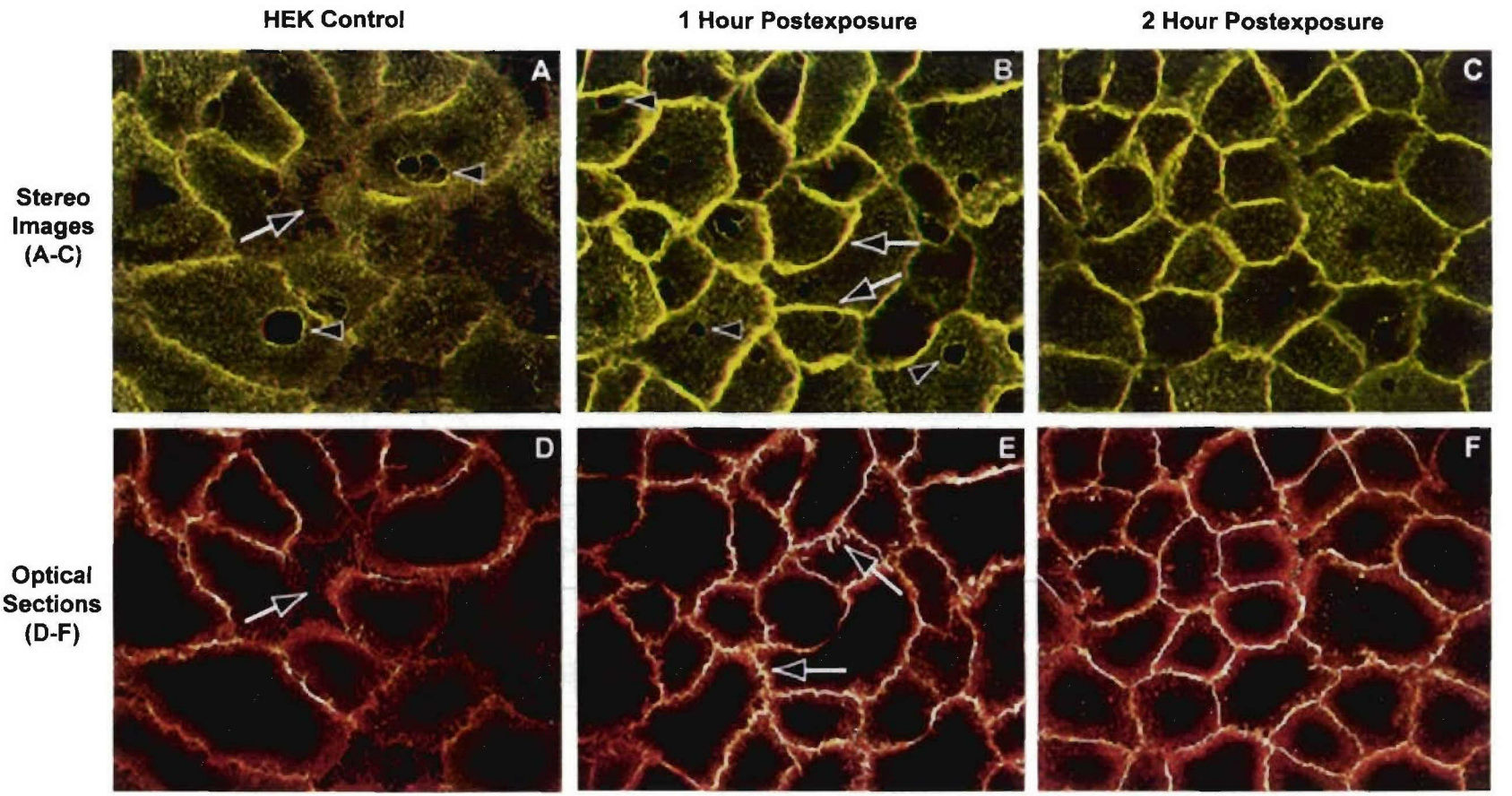

Figure 1. In the HEK control population, stereo image (A) shows how $\alpha_3\beta_1$ integrins cast a veil-like cover of receptors over the raised central nucleus or dome of each cell and down to the rim of basolateral attachments, including the filopodial retraction fibers (arrows, A & D). HEK often contained an open ring or aperture within the cover of $\alpha_3\beta_1$ receptors (arrowheads, A). At 1 h postexposure, stereo image (B) shows there were intense emissions at the HEK margins (arrows) that resulted from lateral compression of adjacent cell membranes and accretion of their $\alpha_3\beta_1$ surface receptors. At the attachment surface, optical section (E) shows loss of retraction fibers (arrows) and decreased functional asymmetry. However, HEK maintained an otherwise characteristic $\alpha_3\beta_1$ surface appearance (B), including the $\alpha_3\beta_1$ aperture (arrowheads). At 2 h postexposure, cells had a geometric, flagstone appearance (C & F); and collapse of the HEK's domed centers was evident (C). Optical section (F) shows complete loss of functional asymmetry and retraction fibers, i.e., loss of polarized mobility. Still, the characteristic $\alpha_3\beta_1$ surface (C) remained relatively intact. All optical sections (D-F) were at 1.4 μm from the HEK attachment surface.

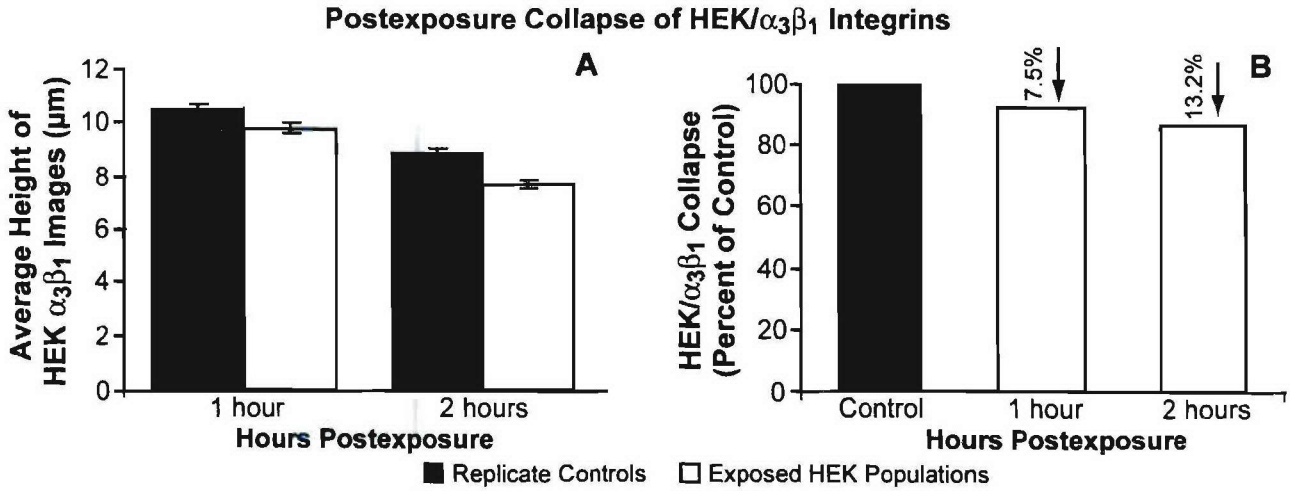

Figure 2. Postexposure collapse of HEK/$\alpha_3\beta_1$ surface integrins was progressive. Graph (A) shows the average height of HEK/$\alpha_3\beta_1$ images (± S.E.M.) decreased at 1 h postexposure from 10.75 ± 0.10 μm to 9.94 ± 0.19 μm, (n = 44), i.e., by 7.5% compared with replicate controls (B). At 2 h postexposure, the average height of HEK/$\alpha_3\beta_1$ images had collapsed from 9.03 ± 0.21 μm to 7.84 ± 0.18 μm (n = 34), i.e., by 13.2% compared with replicate controls.

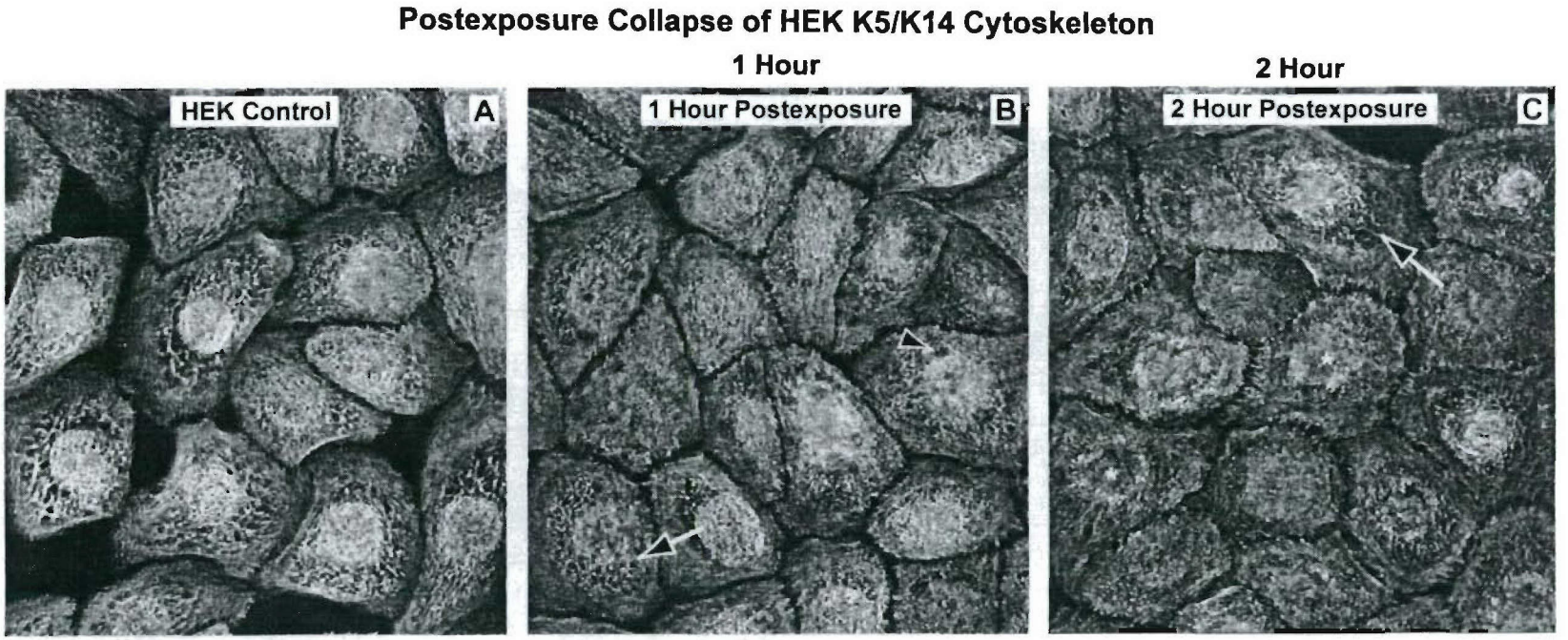

Figure 3. Images from keratin studies showed that postexposure collapse of the K5/K14 cytoskeleton was also progressive and roughly coincident with collapse of the HEK's $\alpha_3\beta_1$ integrin surface. The 1-h collapse of keratin 14 appeared to be more advanced than collapse of the $\alpha_3\beta_1$ surface receptors. However, at 2 h postexposure, the extent of collapse affecting the keratin cytoskeleton and the $\alpha_3\beta_1$ surface receptors appeared to be similar. Previous studies[1] showed that collapse of the K5/K14 cytoskeleton continued to worsen through 6 h of postexposure study.

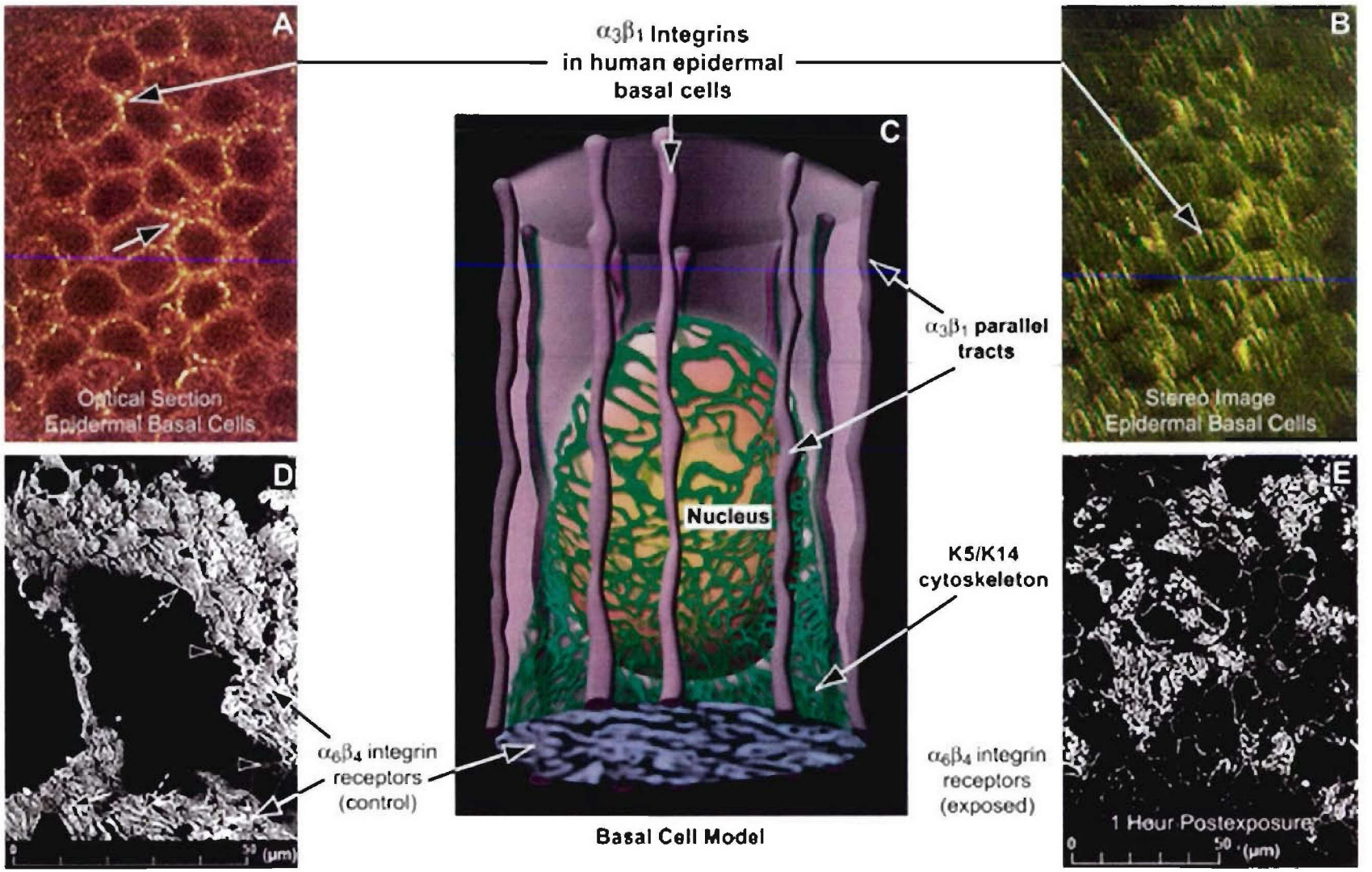

Figure 4. Optical sections of human epidermal basal cells showed $\alpha_3\beta_1$ integrins as a necklace of bright, evenly spaced, punctate units circumscribed around the perimeter of the constituent HEK (A, arrows). Stereo images of the same image field (B) showed that the punctate units were actually cross sections of $\alpha_3\beta_1$-receptor tracts that ran parallel to the long axis of the cells (arrow). Illustration (C) shows how $\alpha_3\beta_1$ parallel tracts were aligned perpendicular to the $\alpha_6\beta_4$ integrin receptors expressed on the ventral surface of polarized basal cells. Exposure to SM disrupts the structural organization and presentation of $\alpha_6\beta_4$ receptors (D and E) and $\alpha_3\beta_1$ receptors (figure 1) and presumably, therefore, disrupts their capacity to stabilize cell-to-cell and cell-to-substrate attachments.